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TECHNICAL MANUSCRIPT 606

CHROMOSOME MAPPING OF PASTEURELLA PSEUDOTUBERCULOSIS BY INTERRUPTED MATING

William D. Lawton
Harold B. Stull

MAY 1970

DEPARTMENT OF THE ARMY
Fort Detrick
Frederick, Maryland

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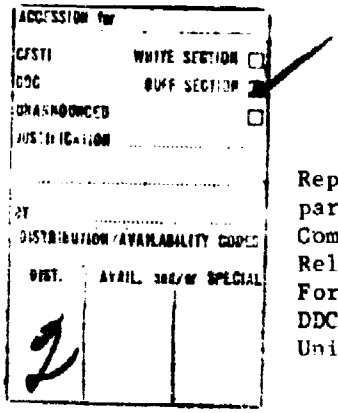

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TECHNICAL MANUSCRIPT 606

CHROMOSOME MAPPING OF *PASTEURELLA PSEUDOTUBERCULOSIS* BY INTERRUPTED MATING

William D. Lawton

Harold B. Stull

Medical Bacteriology Division
BIOLOGICAL SCIENCES LABORATORIES

Project 1B061102B71A

May 1970

ABSTRACT

Pasteurella pseudotuberculosis containing the plasmid F'lac transferred its chromosome from a specific origin in an oriented manner to five multiply auxotrophic strains of P. pseudotuberculosis. In a mating system containing gelatin, glucose, and phosphate buffer, a maximum of 0.1% of the donor cells transferred lead markers. The donor population was counterselected with nalidixic acid. P. pseudotuberculosis containing the plasmids F'CM, F'13, or F'his donated markers in the same order and with approximately the same entry times as did the standard F'lac donor strain, suggesting that F itself has enough homology with a specific region on the chromosome of P. pseudotuberculosis to mobilize the chromosome. We have established the entry time (in minutes after mixing parental cultures) of seven markers as follows: proline (10 minutes); arginine (14 minutes); histidine (14 minutes); threonine (25 minutes); lysine (50 minutes); tyrosine (67 minutes); and tryptophan (77 minutes). The resulting chromosome map shows a marked similarity both in relative order and in distance between markers to the well established chromosome map of Escherichia coli.

CONTENTS

FIGURES

TABLE

I. INTRODUCTION*

Gene transfer by conjugation between auxotrophs of Pasteurella pseudotuberculosis has been demonstrated by Lawton, Morris, and Burrows.[1] Their data were obtained by mixing donor (F'lac) and recipient cells on selective plates and permitting conjugation, transfer, and recombinant colony formation to occur on the plate. Alternative explanations for their results, such as syntrophy or reversion, were not substantiated by experiments showing that (i) replacement of the donor by an F⁻lac⁻ derivative, which would be expected to cross-feed the recipient equally well, resulted in infertility; (ii) separation of parents by a membrane eliminated fertility; and (iii) a large number of recombinants showed unselected donor markers. The extension of the initial observations to classical interrupted mating experiments was hindered by the apparent lack of gene transfer in broth and by the difficulty of preventing the donor strain from remating on the selective plate.

We have solved both of these problems and report here our method of interrupted mating and the resulting preliminary chromosome map of P. pseudotuberculosis.

II. MATERIALS AND METHODS

A. ORGANISMS

All the bacterial strains used were drived from P. pseudotuberculosis strain 32IV obtained from Professor E. Thal, Stockholm. The initial auxotrophs were obtained at the Microbiological Research Establishment (MRE), Porton, England, and are described in the publication of Lawton, Morris, and Burrows.[1] The donor strain used in this study, which was obtained from strain MRE 2027 by isolating a single colony resistant to 1 mg streptomycin sulfate per ml, was designated YsD-20 (cys-5, pth-2, str-50; F'lac). The recipient strains, derived from strain MRE 2205 (met-5, arg-8) after treatment with nitrosoguanidine and subsequent isolation of spontaneous mutants resistant to 20 µg nalidixic acid per ml, were designated YsD-16 (met-5, arg-8, his-50, thr-50, nal-51), YsD-17 (met-5, arg-8, his-50, pro-50, nal-52), YsD-18 (met-5, arg-8, his-50, lys-50, nal-53), YsD-19 (met-5, arg-8, his-50, tyr-50, nal-54), and YsD-21 (met-5, arg-8, his-50, trp-50, nal-55). The designations for our genetic stock strains reflect the current consensus of investigators in the field that the species Pasteurella pseudotuberculosis and Pasteurella

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pestis should be distinguished from other members of the genus Pasteurella. Anticipating the eventual renaming of these species under the genus Yersinia, we have designated all our genetic stocks of P. pseudotuberculosis as Ys- and of P. pestis as Yp-. Thus YsD- has been used for all mutants derived from wild type P. pseudotuberculosis 32IV. The number following the YsD- is merely an isolation number. Numerical genotype designations reflect an agreement between the only two laboratories currently publishing on derived mutants of P. pseudotuberculosis. Dr. T.W. Burrows and co-workers of the Microbiological Research Establishment use numbers 1 through 49 for genotype designation and we use numbers 50 through 99.

B. ABBREVIATIONS

The designation of genotype and phenotype essentially follows the recommendations of Demerec et al.[3] with the additional symbols: pth, double requirement for any purine + thiamine; nal, nalidixic acid.

C. MEDIA

Our selective agar was made as follows: distilled water (1,000 ml); K_2HPO_4 (1.5 g); L-glutamic acid, CalBiochem (0.5 g); 12 N HCl (approximately 0.075 ml to obtain pH 6.8); $MgCl_2 \cdot 6H_2O$ (1.0 g); Bacto agar (9.0 g); after autoclaving, stock solutions were added aseptically to obtain final concentrations of 0.2% glucose (20% stock solution, filtered), 10 µg nalidixic acid per ml (5 mg/ml alkaline stock solution, filtered), and 50 µg of required amino acids per ml (10 mg/ml stock solutions); final pH was 6.7.

Viable counts were made on Difco purple broth base supplemented with 1.5% agar, 1.0% lactose, and 0.0125% triphenyltetrazolium chloride.

D. MATING PROCEDURE

After exploring many of the variables presented in Section III, our final mating procedure was standardized as follows: Recipient cells were grown on Difco blood agar base slants for 21 hours at 26 C. The cells were washed off the surface into 10 ml of 1% Difco gelatin, 0.4% glucose, and 0.001 M sodium phosphate, pH 6.7. The viable count was approximately 3×10^9 cells/ml. Donor cells were grown in Difco brain heart infusion plus 0.1% lactose added aseptically (BHI + lactose) on a shaker at 26 C until the optical density reached 1.0 to 1.1 (650 mµ, Coleman 14 spectrophotometer). This took approximately 23 hours and yielded a viable count of approximately 1×10^9 cells/ml. To achieve a standard inoculum, we added 10% dimethyl sulfoxide to a 23-hour culture, froze it at -60 C, and used 0.07 ml of the thawed cells to inoculate 50 ml

of fresh medium in a 250-ml flask. Three-milliliter portions of donor culture were exposed, with agitation in a petri plate, to a single GE 15-watt ultraviolet bulb at a distance of 61 cm for 60 seconds (approximately 400 ergs/mm^2). Five milliliters of the treated cells were added to 5 ml of BHI + lactose and the diluted sample was shaken very gently in a 50-ml flask at 34 C for 5 hours. The initial viability of approximately 8 x 10^8 cells/ml was reduced approximately 50% after ultraviolet treatment and remained at that number after the subsequent 5-hour incubation.

One milliliter of recipient cells plus 1 ml of donor cells plus 3 ml of 0.5% gelatin, 0.2% glucose, and 0.001 M sodium phosphate, pH 6.7, were placed in a tube and incubated statically in a water bath at 34 C. Thirty minutes after combining recipient and donor cells, we gently added 3 ml of 0.5% gelatin, 0.2% glucose, and 0.1 M sodium phosphate, pH 6.7, and continued the incubation. Greater volumes were used successfully, but always in the proportions above. Periodically, we removed 1-ml samples, blended for approximately 2 seconds at high speed on a Vortex mixer, and plated appropriate samples on various selective agar plates. Samples plated at 0 minutes showed no revertants for most markers and only an average of 10/ml or less for some markers. Dilutions, when necessary, were made in a solution containing 0.01% gelatin, 0.002% glucose, 0.00001 M sodium phosphate, pH 6.7, and 3 µg nalidixic acid per ml. Plates were incubated at 26 C and counted after 3 or 4 days.

III. RESULTS

A. COUNTERSELECTION OF DONOR

The elimination of the donor—to prevent mating on the selective plate—by the classical use of streptomycin or phage was unsatisfactory because the donor strain was resistant to streptomycin and no suitable phages were available. Nalidixic acid was shown by Barbour[3] to inhibit conjugational gene transfer, and we found it effective in eliminating mating on the selective plates. Our five auxotrophic recipient strains were isolated as spontaneous mutants resistant to 20 µg nalidixic acid per ml, but 20 µg/ml in the selective agar yielded a lower number of recombinants than 10, 5, or 3 µg/ml.

B. MEMBRANE MATING

We attempted initially to study the kinetics of marker entry by permitting mating to occur on Millipore membranes on a variety of agar media, periodically removing a membrane, separating the mating pairs by blending, and scoring for recombinant colonies on selective agar containing

nalidixic acid. This technique permitted us to establish a chromosome order for five markers, but the technique was not exact enough to establish unequivocal entry times. During our empirical investigation of a variety of media for mating, we noted a dramatic increase in recombinants when mating took place on the SD agar medium (gelatin, succinate, and salts) used by Landman and Halle[4] for maintaining protoplasts.

C. DEVELOPMENT OF A LIQUID MATING SYSTEM

Because of our past failures to observe chromosome transfer in either tryptic meat broth or in heart infusion broth compared with the successful chromosome transfer observed on SD mating agar, we started by simply removing the agar from the SD mating medium. We obtained low-frequency gene transfer and soon found that this medium could be further simplified by removal of the succinate and most of the salts without lowering the gene transfer frequency. Our conjugation procedure at that stage consisted of mixing 1 ml of the recipient culture, 1 ml of the donor culture, and 3 ml of 1% gelatin, 0.2% glucose, and 0.001 M potassium phosphate buffer, pH 6.7. Figure 1 illustrates our initial results in studying the entry of the marker $\underline{arg}^+$. The most apparent problem appeared to be the loss of $\underline{arg}^+$ recombinants after 90 minutes of mating, correlated with and perhaps caused by the rapidly declining pH of the system. Our attempt to control the pH simply by increasing the molarity of the buffer demonstrated that increased levels of buffer inhibited the conjugation system. Figure 2 shows the inhibition curves obtained with sodium phosphate buffer; essentially the same inhibition was also obtained with potassium phosphate or sodium malate buffer. The level of buffer required to maintain the pH above 6.0 was 0.05 M, and, at that level, gene transfer was essentially stopped.

On the basis of the hypothesis that the inhibition by high concentrations of buffer was preventing effective contacts, we permitted the donor and recipient cells to form pairs in the usual 1% gelatin, 0.2% glucose, and 0,001 M sodium phosphate, pH 6.7, for 30 minutes, and then added 3 ml of 1% gelatin, 0.2% glucose, and 0.05 M sodium phosphate, pH 6.7. As shown in Figure 3, this procedure did not inhibit chromosome transfer. On the contrary, it maintained the pH and permitted the recovery of an increased number of $\underline{arg}^+$ recombinants even 120 minutes after mating. The control, to which 3 ml of 1% gelatin, 0.2% glucose, and 0.001 M sodium phosphate, pH 6.7, were added after 30 minutes, showed the expected decline of pH and decrease of $\underline{arg}^+$ recombinants after 75 minutes.

Variability in the mating system seemed to be correlated with small variations in the method of preparing the donor cells. For this reason, we investigated several of these procedures using the transfer of the $\underline{arg}^+$ marker from YsD-20 to YsD-21 as a measure of the best conditions to prepare the donor strain for mating. We found that: (i) 23-hour incubation of the donor strain was better than 20, 21, 22, or 24 hours; (ii) 60-second ultraviolet irradiation was better than 0, 20, 40, 100, or 120 seconds; mitomycin C showed a similar donor-enhancing effect, but was not as efficient as was UV;

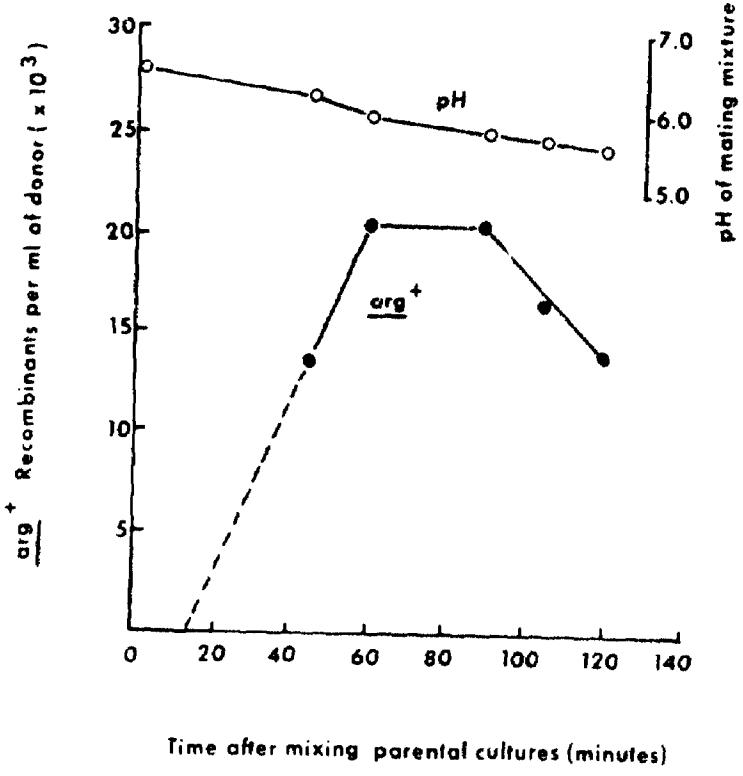


FIGURE 1. Entry of arg+ from YsD-20 (F'lac) to YsD-21 (F⁻).

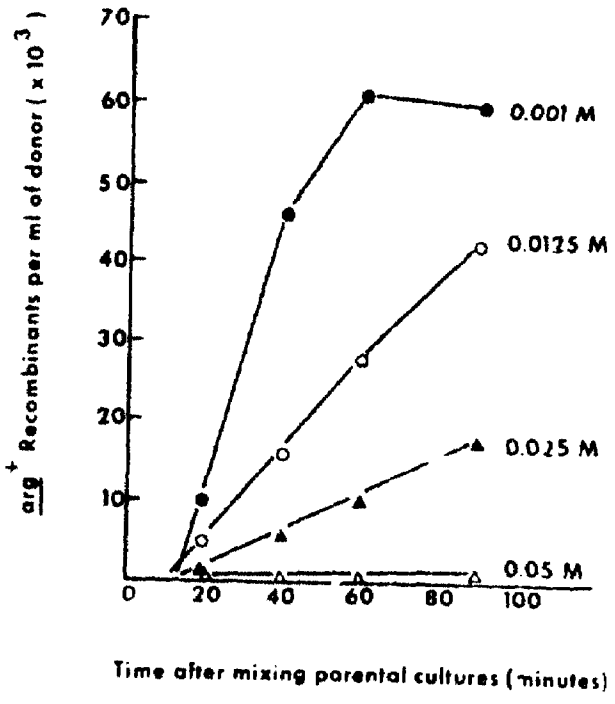


FIGURE 2. Effect of Adding Sodium Phosphate Buffer, pH 6.7, on the Transfer of arg+ from YsD-20 (F'lac) to YsD-21 (F⁻).

(iii) after UV irradiation, shaking the donor strain at 34 C for 5 hours was better than for 3, 7, or 24 hours; (iv) the 5-hour "expression" period as well as the mating was better at 34 C than at 37 C; (v) for mating, 0.2% glucose was better than 1.0% glucose, 0.5% gelatin was equivalent to 1% or 2%, and better than 0.25%; and (vi) different sources of gelatin resulted in large differences in mating efficiency.

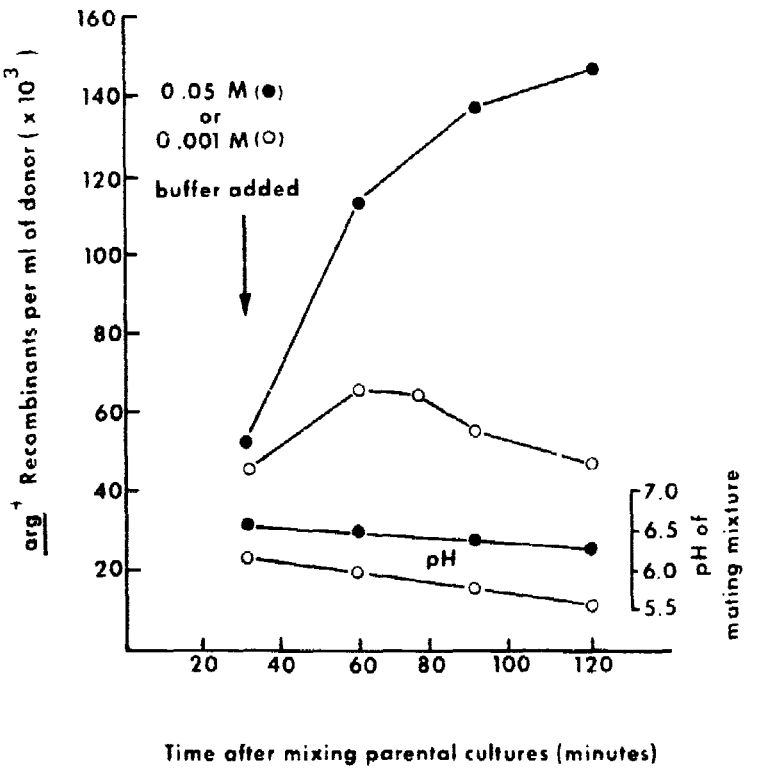


FIGURE 3. Effect of Adding Sodium Phosphate Buffer, pH 6.7, after 30-Minute Contact Period on the Transfer of arg$^+$ from YsD-20 (F'lac) to YsD-21 (F$^-$).

After we established optimal conditions for mating, each of the five auxotrophic recipients was mated with the donor strain. Figures 4 through 8 show the results of a mating experiment with each recipient. This type of experiment was performed several times with each recipient and provided us with the entry times shown in Table 1.

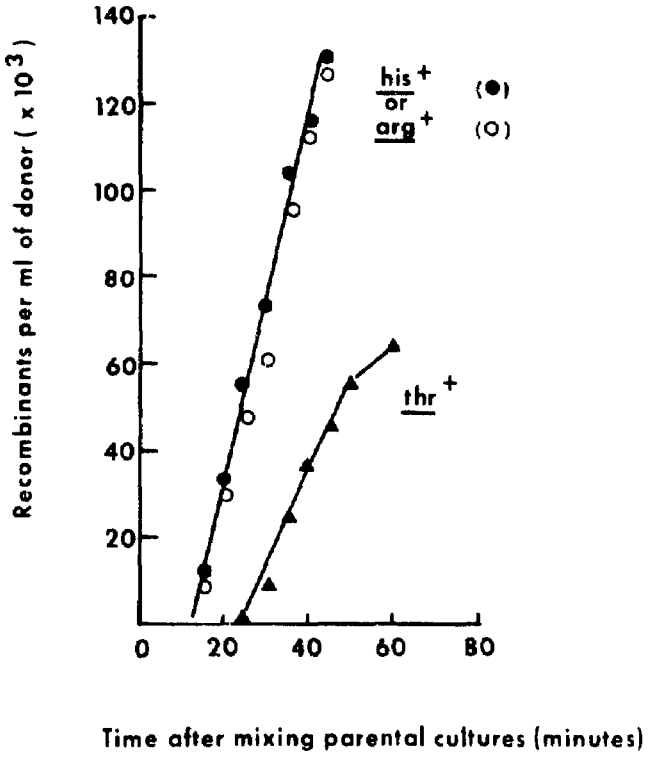


FIGURE 4. Interrupted Mating of YsD-20 (F'lac) and YsD-16 (F^-).

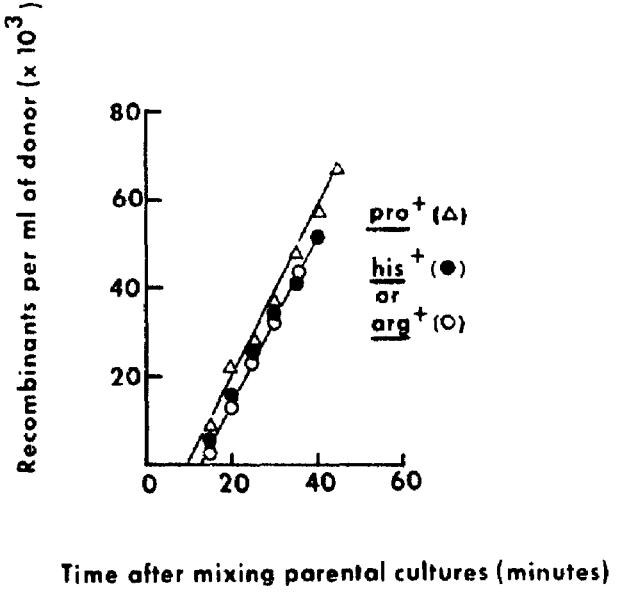


FIGURE 5. Interrupted Mating of YsD-20 (F'lac) and YsD-17 (F^-).

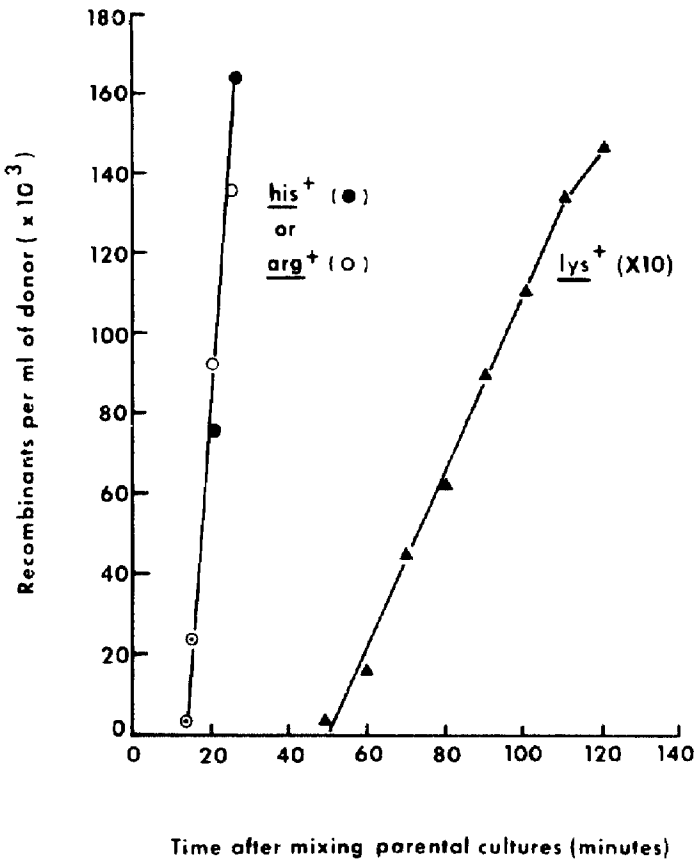


FIGURE 6. Interrupted Mating of YsD-20 (F'lac) and YsD-18 (F$^-$).

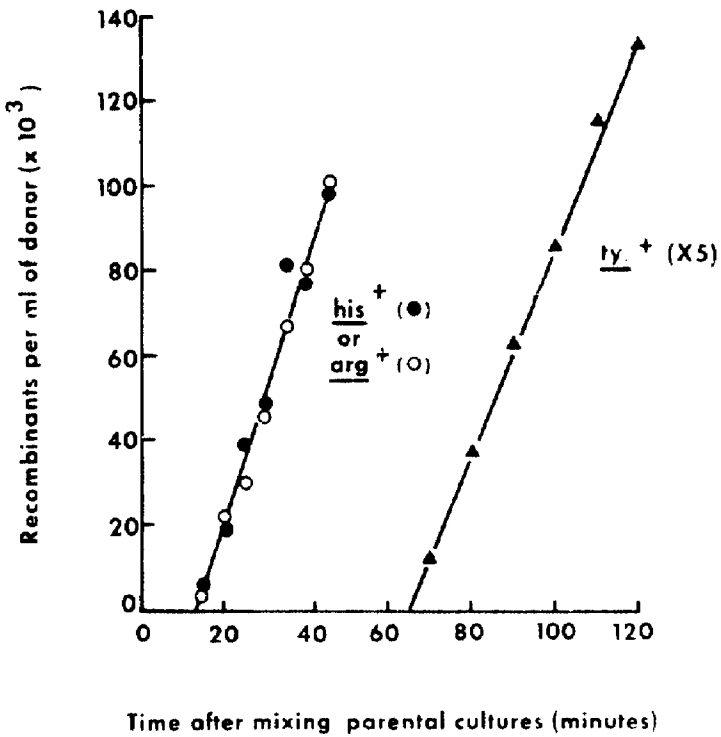


FIGURE 7. Interrupted Mating of YsD-20 (F'lac) and YsD-19 (F$^-$).

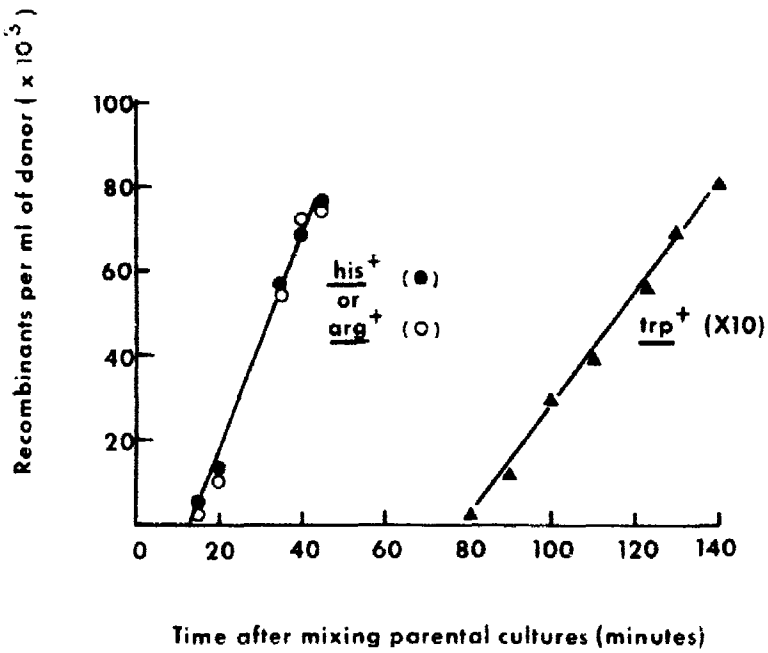


FIGURE 8. Interrupted Mating of YsD-20 (F'lac) and YsD-21 (F^-).

TABLE 1. TIME OF ENTRY OF MARKERS FROM P. PSEUDOTUBERCULOSIS (YsD-20)

Marker	Average Entry Time, minutes	Range of Entry Times, minutes	Number of Experiments to Obtain Average
pro	10.5	7-14	15
arg	13.7	13-14	13
his	13.7	13-14	29
thr	24.8	24-26	5
lys	50.4	48-52	5
tyr	66.8	62-71	4
trp	76.6	70-81	8

D. METHIONINE MARKER

Although all of the recipient strains were derived from the *met*$^-$ *arg*$^-$ MRE 2205 strain, the *met*$^-$ marker proved to be unsatisfactory for obtaining clear-cut results. None of the five recipient strains grew in the absence of methionine, but they did produce background growth and, after prolonged incubation, tiny colonies appeared on the plates selective for *met*$^+$ recombinants that proved to be *met*$^-$ clones being fed methionine. Our results indicated that the *met* marker was closely linked to *trp*, but we have postponed including the *met* locus in the chromosome map until better *met*$^-$ auxotrophs are investigated.

E. DETERMINING ORDER OF THE *arg*-*his* REGION

The entry times of the *arg* and the *his* markers were too close to determine their order of entry. Of 518 *arg*$^+$ recombinants (obtained 40 to 120 minutes after mating YsD-20 and YsD-17), 69% were also *his*$^+$; of 454 *his*$^+$ recombinants, 77% were also *arg*$^+$, suggesting that *arg* enters before *his*. We performed a three-point cross using the recipient strain YsD-17 (*pro*$^-$ *arg*$^-$ *his*$^-$), and selected for double and triple recombinants. Of 211 *pro*$^+$ *his*$^+$ recombinants, 11 (5.2%) were *arg*$^-$; of 269 *pro*$^+$ *arg*$^+$ recombinants, 58 (21.6%) were *his*$^-$. These values were obtained by subtracting the number of *pro*$^+$ *arg*$^+$ *his*$^+$ recombinants from the number of *pro*$^+$ *his*$^+$ or *pro*$^+$ *arg*$^+$ obtained on selective plates. To confirm our observation, in a separate experiment, we picked and purified a number of *pro*$^+$ *his*$^+$ and *pro*$^+$ *arg*$^+$ recombinants and tested their requirement for the third marker. Of 76 *pro*$^+$ *his*$^+$ recombinants, six (7.9%) were *arg*$^-$; of 84 *pro*$^+$ *arg*$^+$ recombinants, 23 (27.4%) were *his*$^-$. Since the recombinant class *pro*$^+$ *arg*$^-$ *his*$^+$ was approximately four times less frequent than the class *pro*$^+$ *arg*$^+$ *his*$^-$, we concluded that the correct order was *pro*-*arg*-*his*.

F. MATING EFFICIENCY

To determine the maximum number of possible recombinants, we assayed the appearance of *pro*$^+$, *arg*$^+$, and *his*$^+$ recombinants for an extended mating period. Approximately 3 hours after mixing parental cultures, each of the three types reached a plateau at approximately 2×10^5 recombinants per ml of donor, indicating that a maximum of 0.1% of the donor population transferred their lead markers. Several single colony isolates from the donor population transferred markers with the same efficiency as the parent population. The number of viable donor and recipient cells per ml of mating mixture did not change during 3½ hours of mating.

C. CHROMOSOME MOBILIZATION BY OTHER F' PLASMIDS

A spontaneously occurring *lac*$^-$ (F^-) clone was isolated from the donor strain YsD-20 and reinfected with the plasmid F'CM by mating with *E. coli* K57[5] or with the plasmid F'13 by mating with *E. coli* W3747.[6] Also, the nalidixic acid - sensitive strain from which we obtained the recipient YsD-17 was infected with the plasmid F'*his* by mating with *E. coli* X^{823} (provided by Roy Curtiss III*). Each of these three different *P. pseudotuberculosis* F' strains donated the markers *arg* and *thr* or *trp* in the same order and with approximately the same entry times as did YsD-20 (F'*lac*).

IV. DISCUSSION

Our data establish, for the first time, a chromosome map of *P. pseudotuberculosis* that shows more than the relative order of markers. Morris and Burrows[7] published a chromosome map of *P. pseudotuberculosis* based on a frequency analysis of multiple unselected markers. Our entry times support their conclusion that *arg*-8 and *his*-8 enter early, but our other markers were different from the other markers they used, so no other direct comparisons are possible.

Although our map is based on matings with five different recipients, each of the five accepted the *arg*-8 and *his*-8 genes from the donor at the same entry time, making it possible to compare all recipients for the entry of their unique markers.

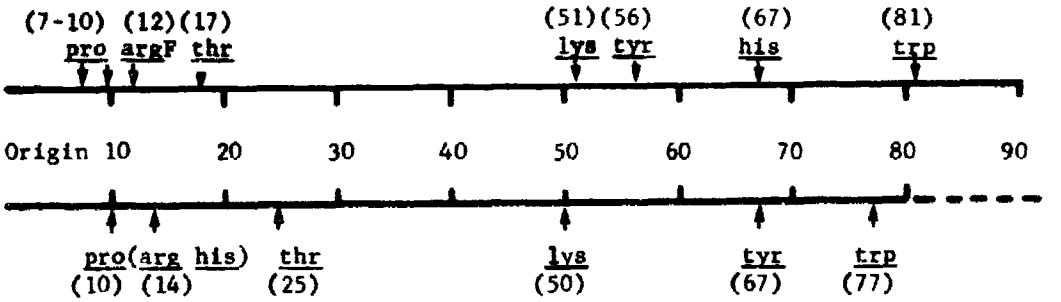
Since each marker had a specific entry time, we presume that the F'*lac* plasmid can mobilize the chromosome of *P. pseudotuberculosis* at a specific origin and is responsible for its transfer in a specific direction. In our system, approximately 0.1% of the male cells donated lead markers. Chromosome transfer did not appear to result from the presence of a few Hfr cells in our male population, because several single colony isolates showed the same gene transfer frequency as the parent male population. Also, the donor-enhancing effect shown by ultraviolet irradiation or by mitomycin C treatment suggested that F'*lac* in *P. pseudotuberculosis* may function as Evenchik, Stacey, and Hayes[8] found an F' factor to function in *E. coli*. They suggested a mechanism whereby the excision of single-stranded fragments of the bacterial chromosome, during the repair of UV damage, facilitates pairing with homologous regions of the complementary sex factor. Since the presence of four different F' plasmids in *P. pseudotuberculosis* all resulted in the transfer of the chromosome from the same origin, it appears

* Oak kidge National Laboratory, Oak Ridge, Tennessee.

that a specific region in the chromosome of P. pseudotuberculosis has close enough homology with the F factor itself to permit recombination and subsequent oriented transfer of the chromosome.

The availability of a partial chromosome map for P. pseudotuberculosis permits us to make comparisons with the map of E. coli.[9] Based on a theoretical origin at minute 17 on the E. coli map and a counterclockwise direction of transfer, a comparison of the E. coli map and the P. pseudotuberculosis map can be diagrammed as follows (all numbers represent minutes after mixing parental cultures):

E. coli



P. pseudotuberculosis

The markers pro, arg, thr, lys, tyr, and trp are in the same order in both genera; the distances between the markers seem to be approximately the same for some markers but different by several minutes for others. The marker his did not conform to the pattern of similarity.

The value of attempting to compare the P. pseudotuberculosis chromosome map with the well established map of E. coli is in the possible predictive value to guide future mapping studies with Pasteurella. The comparison suggests that the chromosome of P. pseudotuberculosis may be approximately the same length as that of E. coli and that the rate of chromosome transfer may be similar. We can predict from this comparison that the galactose marker may be very close to the terminus of the P. pseudotuberculosis chromosome and, if this proves correct, we should have the greatest chance of success in finding an integrated F, or Hfr type, among gal^+ recombinants.

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13. ABSTRACT

Pasteurella pseudotuberculosis containing the plasmid F'lac transferred its chromosome from a specific origin in an oriented manner to five multiply auxotrophic strains of P. pseudotuberculosis. In a mating system containing gelatin, glucose, and phosphate buffer, a maximum of 0.1% of the donor cells transferred lead markers. The donor population was counterselected with nalidixic acid. P. pseudotuberculosis containing the plasmids F'CM, F'13, or F'his donated markers in the same order and with approximately the same entry times as did the standard F'lac donor strain, suggesting that F itself has enough homology with a specific region on the chromosome of P. pseudotuberculosis to mobilize the chromosome. We have established the entry time (in minutes after mixing parental cultures) of seven markers as follows: proline (10 minutes); arginine (14 minutes); histidine (14 minutes); threonine (25 minutes); lysine (50 minutes); tyrosine (67 minutes); and tryptophan (77 minutes). The resulting chromosome map shows a marked similarity both in relative order and in distance between markers to the well established chromosome map of Escherichia coli.

14. Key Words

Pasteurella pseudotuberculosis	Membrane mating	Escherichia coli
Chromosome map	Gene transfer	Auxotrophs

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